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Bcl-x_L Regulates the Membrane Potential and Volume Homeostasis of Mitochondria

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Summary

Mitochondrial physiology is disrupted in either apoptosis or necrosis. Here, we report that a wide variety of apoptotic and necrotic stimuli induce progressive mitochondrial swelling and outer mitochondrial membrane rupture. Discontinuity of the outer mitochondrial membrane results in cytochrome c redistribution from the intermembrane space to the cytosol followed by subsequent inner mitochondrial membrane depolarization. The mitochondrial membrane protein Bcl-x_L can inhibit these changes in cells treated with apoptotic stimuli. In addition, Bcl-x_L-expressing cells adapt to growth factor withdrawal or staurosporine treatment by maintaining a decreased mitochondrial membrane potential. Bcl-x_L expression also prevents mitochondrial swelling in response to agents that inhibit oxidative phosphorylation. These data suggest that Bcl-x_L promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria.

Introduction

Apoptosis, or programmed cell death (PCD), is an evolutionarily conserved mechanism essential for the development and maintenance of tissue homeostasis in multicellular organisms (for review, see White, 1996; Yang and Korsmeyer, 1996). Apoptosis results from the action of a genetically encoded suicide program that leads to a series of characteristic morphological and biochemical changes. These changes include activation of caspases (formerly known as the ICE-like proteases), mitochondrial depolarization, cell volume loss, chromatin condensation, and nucleosomal DNA fragmentation. Members of the *bcl-2* gene family encode proteins that function either to promote or to inhibit apoptosis. Antiapoptotic members such as Bcl-2 and Bcl-x_L prevent PCD in response to a wide variety of stimuli. Conversely, proapoptotic proteins, exemplified by Bax and Bak, can accelerate death and in some instances are sufficient to cause apoptosis independent of additional signals. However, the biochemical mechanism by which Bcl-2-related proteins regulate apoptosis has yet to be determined.

Various initiators of PCD have been used to study apoptosis. It appears that the downstream biochemical events in apoptosis are the same regardless of the initial death stimulus. This suggests that all apoptotic signals converge on a common pathway that results in cell suicide. Several different biochemical changes have been proposed to be the essential event that commits a cell to undergo apoptosis. These events include the generation of reactive oxygen species (ROS), calcium flux, caspase activation, loss of mitochondrial membrane potential, and cytochrome c redistribution. All of these biochemical perturbations can result from alterations in mitochondrial function. Mitochondria are primary generators of ROS (Boveris and Chance, 1973) and important storage sites for intracellular calcium (Gunter et al., 1994). In addition, at least two resident mitochondrial proteins, apoptosis-initiating factor (AIF) and cytochrome c, have been implicated in the activation of caspases (Liu et al., 1996; Susin et al., 1996).

Bcl-2 expression has been shown to affect all of the above biochemical changes, yet it remains unclear if this is a direct function of the protein or merely a consequence of Bcl-2 blocking an earlier step in the common death pathway. One common feature of Bcl-2-related proteins is that these proteins are localized to the outer mitochondrial, outer nuclear, and endoplasmic reticular membranes as a result of a carboxy-terminal membrane anchor (Yang and Korsmeyer, 1996). Furthermore, there is evidence that Bcl-2 proteins exert their function at the organelles to which they are distributed. For example, Bcl-2 can prevent the depletion of endoplasmic reticulum calcium stores upon thapsigargin treatment (Lam et al., 1994). Bcl-2 can also block mitochondrial permeability transition induced by treatment of isolated mitochondria with a variety of agents (Zamzami et al., 1996). Additionally, in cell free systems, Bcl-2 can prevent the release of caspase activators from mitochondria (Kluck et al., 1997), and the proapoptotic member Bax can cause death through direct mitochondrial effects (Xiang et al., 1996).

The three-dimensional structure of Bcl-x_L, an antiapoptotic member of the Bcl-2 family, has been determined. The structure of Bcl-x_L is closely related to the pore-forming domains of bacterial toxins such as diphtheria toxin and the colicins (Muchmore et al., 1996). In a manner that is analogous to these bacterial proteins, Bcl-x_L can form a functional ion channel in synthetic lipid membranes. The Bcl-x_L channel has multiple conductance states, is cation selective, and inserts in a pH-sensitive manner (Minn et al., 1997). Bcl-2 and Bax can also form ion channels in synthetic membranes (Antonsson et al., 1997; Schendel et al., 1997). The potential for Bcl-2 proteins to form an ion channel in intracellular membranes suggests that they may function to regulate the permeability of the membranes to which they distribute.

To clarify how Bcl-x_L acts to regulate mitochondria function, we investigated alterations in mitochondrial physiology that follow apoptotic stimuli to determine which, if any, of the alterations can be affected by Bcl-x_L expression. We demonstrate that in response to death stimuli, the outer mitochondrial membrane is physically disrupted as a result of inner mitochondrial membrane hyperpolarization and matrix swelling. Loss of the physical integrity of the outer mitochondrial membrane results

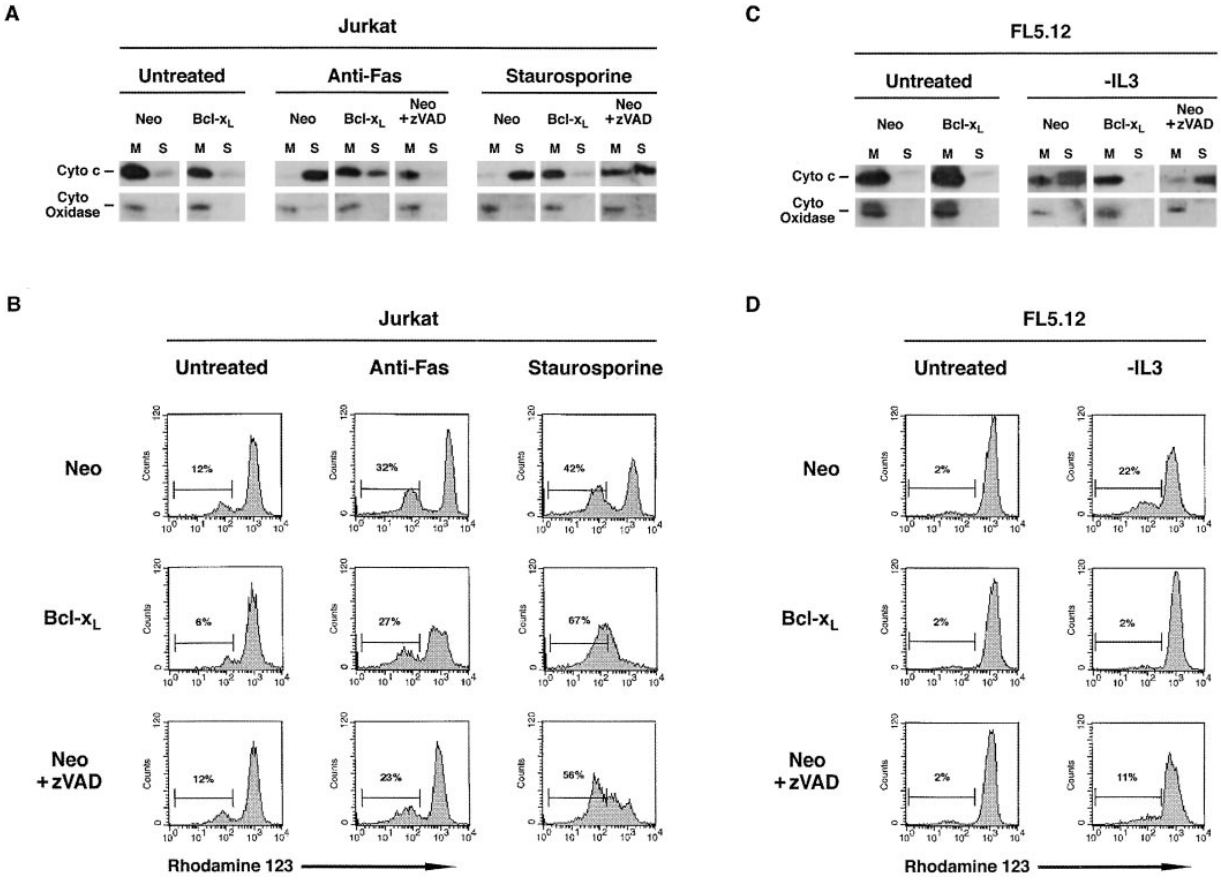

Figure 1. Bcl-x_L Prevents both the Redistribution of Cytochrome c and the Subsequent Loss of Mitochondrial Membrane Potential in Cells Undergoing Apoptosis

(A) Bcl-x_L- and control-transfected (Neo) Jurkat cells were treated for 18 hr with anti-Fas antibody or staurosporine in the presence or absence of zVAD as indicated. After 18 hr, the cells were mechanically lysed and separated into mitochondrial (M) and S100 (S) fractions. The amount of cytochrome c and cytochrome oxidase (subunit IV) present in each fraction was determined by Western blot analysis.

(B) Rh123 fluorescence profiles of Jurkat cells treated as specified in (A) and analyzed by flow cytometry. The percentage of cells falling within the range of Rh123 fluorescence indicative of depolarized cells is shown.

(C) IL-3-dependent FL5.12 cells stably transfected with either Bcl-x_L or a control vector (Neo) were deprived of IL-3 in the presence or absence of zVAD as indicated. After 18 hr, mitochondrial (M) and S100 (S) fractions were analyzed for cytochrome c and cytochrome oxidase by Western blot.

(D) Rh123 fluorescence profiles of FL5.12 cells treated as indicated in (C).

in the redistribution of intermembrane proteins to the cytosol while maintaining, at least transiently, the ability of the inner membrane to carry out oxidative phosphorylation. Bcl-x_L functions to prevent the loss of outer membrane integrity by preventing both membrane hyperpolarization and mitochondrial swelling in response to a diverse set of stimuli. These data suggest that Bcl-x_L may function as an ion channel in the outer membrane to prevent osmotic swelling of mitochondria.

Results

Bcl-x_L Expression Prevents Cytochrome c Redistribution and Subsequent Mitochondrial Depolarization during Apoptosis

Mitochondria appear to play an important role in the early events of apoptosis. Both mitochondrial depolarization and the loss of cytochrome c from the mitochondrial intermembrane space have been proposed as early central events in apoptotic cell death. Whether cytochrome c release occurs prior to the loss of mitochondrial membrane potential or as a result of the loss of inner membrane potential remains controversial (Kluck et al., 1997; Susin et al., 1997; Yang et al., 1997). Since the cell survival protein Bcl-x_L has been shown to localize to the outer mitochondrial membrane, the effects of Bcl-x_L expression on cytochrome c redistribution and mitochondrial membrane depolarization in response to several different apoptotic stimuli were investigated. For comparison, the ability of the caspase inhibitor zVAD-fmk (zVAD) to affect these changes was also examined.

Apoptosis was induced in human Jurkat T cells stably transfected with either Bcl-x_L or a control vector (Neo) by treatment with either an anti-Fas antibody or staurosporine (Figure 1). The subcellular localization of cytochrome c, which normally resides in the mitochondrial intermembrane space, and the mitochondrial membrane potential were assessed at various times following the apoptotic stimuli. At 18 hr, control-transfected cells that

were treated with either an anti-Fas antibody or staurosporine remained >50% alive as measured by propidium iodide (PI) exclusion (data not shown). However, after subcellular fractionation, nearly all of the cytochrome c partitioned with the S100 (cytosolic) fraction (Figure 1A). In contrast, the inner membrane protein cytochrome oxidase remained in the mitochondrial fraction under all conditions tested. Redistribution of cytochrome c could be observed as early as 6 hr following either death stimulus (data not shown). Despite this redistribution of cytochrome c, >50% of the cells maintained a mitochondrial membrane potential as measured by rhodamine-123 (Rh123) fluorescence (Figure 1B). This demonstrates that cytochrome c release from the mitochondria fraction precedes the loss of mitochondrial membrane potential in cells undergoing apoptosis.

In Bcl-x_L-transfected cells, significantly less cytochrome c redistribution occurred in response to either stimulus (Figure 1A). As in control-transfected cells, Bcl-x_L-expressing cells maintained a mitochondrial membrane potential following treatment with an anti-Fas antibody (Figure 1B). However, the majority of Bcl-x_L cells had a decrease in mitochondrial membrane potential in response to staurosporine. Despite this, staurosporine-treated Bcl-x_L cells were >80% viable as measured by PI exclusion. Virtually all of the control-transfected cells with a lower mitochondrial membrane potential were permeable to PI (data not shown).

Bcl-x_L has recently been proposed to regulate caspase activation (Chinnaiyan et al., 1997). Bcl-x_L might therefore maintain mitochondrial integrity as an indirect consequence of caspase inhibition. To test this possibility, the ability of the caspase inhibitor zVAD to mimic the effects of Bcl-x_L expression was examined. The addition of zVAD had no effect on either the mitochondrial membrane potential or the subcellular localization of cytochrome c in untreated cells (Figures 1B and 1D; data not shown). zVAD appeared to be a more potent inhibitor of Fas-induced death than Bcl-x_L. zVAD could completely impede cytochrome c release and the loss of mitochondrial membrane potential in both control and Bcl-x_L cells (Figures 1A and 1B; data not shown). This may reflect the established role of caspase 8 (also known as FLICE, MACH, and Mch5) as a required upstream signaling molecule in Fas-mediated death (Fraser and Evan, 1996). In contrast, staurosporine-induced cytochrome c release was delayed but not inhibited by the same dose of zVAD (Figure 1A). zVAD-treated control transfectants that had a decreased membrane potential following staurosporine treatment were permeable to PI (data not shown).

Cytochrome c redistribution and changes in mitochondrial membrane potential were also examined following growth factor withdrawal in IL-3-dependent FL5.12 cells, a murine pro-B cell line. In control-transfected FL5.12 cells, most of the cytochrome c redistributed to the cytosol after 18 hr of IL-3 deprivation (Figure 1C), while a much smaller percentage of cells had lost mitochondrial membrane potential as measured by Rh123 (Figure 1D). Bcl-x_L expression prevented both cytochrome c redistribution and mitochondrial membrane depolarization. In contrast, zVAD treatment could not prevent either cytochrome c redistribution or mitochondrial membrane depolarization in control transfectants withdrawn from IL-3. Thus, cytochrome c redistribution from mitochondria is an early apoptotic event that precedes mitochondrial membrane depolarization. Bcl-x_L expression functions to inhibit both of these events. In at least some forms of cell death, the ability of Bcl-x_L to regulate these mitochondrial events cannot be mimicked by caspase inhibition.

Loss of Outer Mitochondrial Membrane Integrity during Apoptosis

Although the above data suggest that cytochrome c loss from the intermembrane space reflects an early event in apoptosis, the loss of cytochrome c from the mitochondria could have occurred during cell fractionation. To determine if the cytochrome c redistribution detected by cell fractionation reflects the ability of holo-cytochrome c to pass through a compromised outer mitochondrial membrane in intact cells, FL5.12 cells were assayed for the ability of cytosolic cytochrome c to gain access to cytochrome oxidase on the inner mitochondrial membrane. Holo-cytochrome c was introduced into the cytosol by transient plasma membrane permeabilization. The ability of the outer mitochondrial membrane to exclude added cytochrome c was assessed by measuring the ability of cytochrome c to transfer electrons to cytochrome oxidase as assayed by oxygen consumption. While the introduction of cytochrome c did not change the rate of oxygen consumption in untreated or Bcl-x_L-protected cells, an immediate and consistent increase in the consumption of oxygen was observed in control-transfected cells deprived of IL-3 (Figure 2A). Thus, the outer mitochondrial membrane is bidirectionally permeable to cytochrome c diffusion during apoptosis. Bcl-x_L expression maintains the impermeability of the outer membrane to holo-cytochrome c following IL-3 withdrawal.

Physical Disruption of the Outer Mitochondrial Membrane during Apoptosis

Cytochrome c redistribution could occur as the result of either a specific transport event or a physical disruption of the outer mitochondrial membrane. To exclude the possibility that cytochrome c is specifically transported in either direction across the outer mitochondrial membrane in cells undergoing apoptosis, the ability of a much larger (160 kDa) protein to gain access to the intermembrane space was examined using an antibody to cytochrome oxidase subunit IV. The cytochrome oxidase subunit IV is located on the inner mitochondrial membrane facing the intermembrane space (Tsukihara et al., 1996). Mitochondria isolated from control- and Bcl-x_L-transfected cells grown in the presence of IL-3 bound the cytochrome oxidase antibody to the same extent as an isotype-matched control antibody (Figure 2B). The failure of the cytochrome oxidase antibody to show specific staining suggests that the outer mitochondrial membrane is intact. In contrast, there is enhanced and specific staining with the cytochrome oxidase antibody of the mitochondria isolated from control-transfected cells that have been deprived of IL-3 for 18 hr (Figure 2B). Mitochondria from Bcl-x_L transfectants

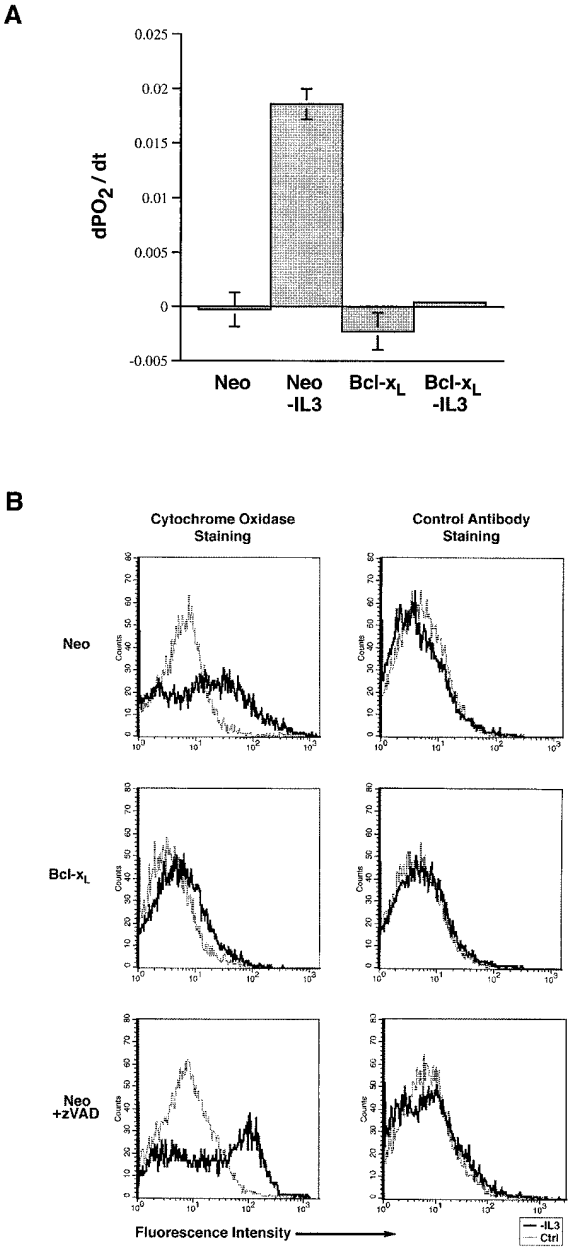

Figure 2. Bcl-x_L Prevents Cytochrome c Redistribution by Maintaining the Integrity of the Outer Mitochondrial Membrane

(A) Cytochrome c was introduced into the cytosol of Bcl-x_L- and control-transfected (Neo) FL5.12 cells cultured for 18 hr in the presence or absence (−IL3) of IL-3 by transient permeabilization of the plasma membrane with saponin. Oxygen consumption was measured using a respirometer containing an oxygen-sensitive electrode. The derivative of the fall in oxygen tension over time (mean ± SD, n = 3; expressed in units of Torr/s) corrected for autooxidation is graphed.

(B) Antibody staining of isolated mitochondria from control- and Bcl-x_L-transfected FL5.12 cells cultured for 18 hr in the presence (Ctrl) or absence (−IL3) of IL-3, with or without zVAD as indicated. Mitochondria were incubated with either a monoclonal antibody to cytochrome oxidase subunit IV or an isotype-matched control. Antibody binding was determined using an FITC-conjugated secondary antibody and flow cytometric analysis.

deprived of IL-3, but not those from zVAD-treated control transfectants, failed to stain with the cytochrome oxidase antibody above the level observed for the isotype-matched control.

To explore this issue further, electron microscopy (EM) of Jurkat cells treated with an anti-Fas antibody was performed. Cells were fixed at a time point when >70% of the cells excluded PI. Cells in the early stages of apoptosis were identified by the absence of nuclear chromatin condensation (Figure 3). Compared to untreated cells, the mitochondria from cells treated with anti-Fas antibody appeared larger and contained fewer cristae, an indication of mitochondrial swelling. Examination of the membrane structure of these swollen mitochondria revealed sites of outer membrane discontinuity, while the inner membrane appeared intact. Similar mitochondrial changes were observed by EM following IL-3 withdrawal in FL5.12 cells, and the changes in both cell types were inhibited by Bcl-x_L expression (data not shown).

Mitochondrial Swelling/Hyperpolarization May Promote the Loss of Outer Membrane Integrity

The EM studies suggest that mitochondrial swelling occurs early in the apoptotic process and contributes to the disruption of the outer mitochondrial membrane. Stereological analysis of the EM data described above confirmed that the mitochondria from cells treated with an apoptotic stimulus exhibit statistically larger volumes than untreated cells ($p < 0.05$) (data not shown). To confirm that the observed mitochondrial swelling was not the result of an EM fixation artifact, the size of isolated mitochondria from cells undergoing apoptosis was measured directly. Mitochondria were isolated from FL5.12 cells cultured for 18 hr in the presence or absence of IL-3, and their size was individually determined by forward angle light scattering (Figure 4). Forward angle light scattering is a direct measure of particle size (Allman et al., 1990). The mitochondria analyzed retained the ability to take up Rh123, confirming that the particles being assessed were mitochondria that maintained a membrane potential. Mitochondria isolated from control-transfected cells deprived of IL-3 demonstrated an increased forward scatter compared to cells grown with IL-3. Mitochondria from Bcl-x_L cells deprived of IL-3 were not increased in size compared to those from cells continuously cultured in IL-3. zVAD treatment of control transfectants failed to protect the mitochondria from swelling in response to either IL-3 deprivation or staurosporine treatment (Figure 4; data not shown).

To verify that an increase in matrix volume occurs physiologically in the absence of fixation or cellular disruption, the accumulation of Rh123, a membrane-permeant, cationic fluorescent dye was examined more closely. The dye fluorescence is proportional to the absolute amount of Rh123, which partitions to the mitochondria according to the Nernst equation: $\Delta\psi = c \cdot \ln([Rh123]_{in}/[Rh123]_{out})$, where $\Delta\psi$ is the potential across the membrane, [Rh123] is the concentration of Rh123 on the indicated side of the membrane, and c is a constant (Lemasters et al., 1987). Thus, Rh123 fluorescence is

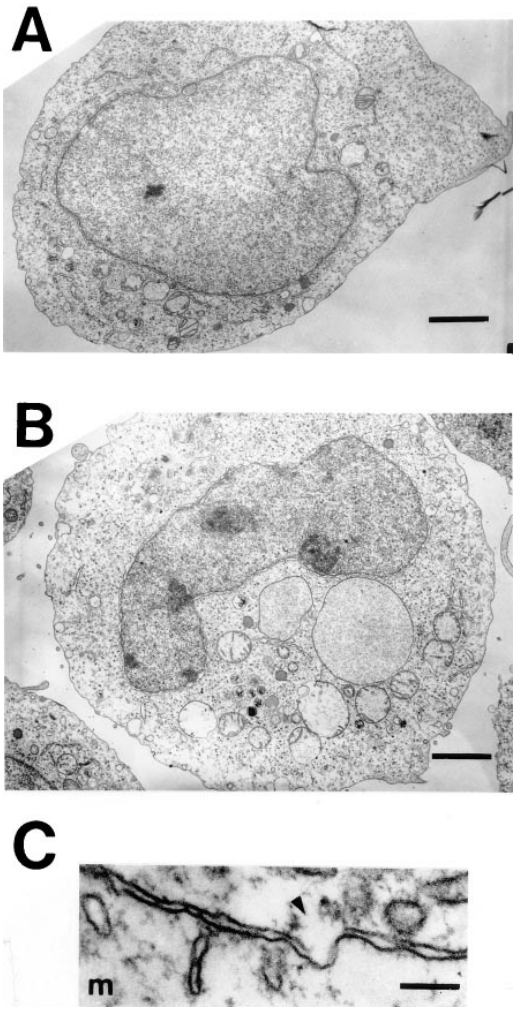

Figure 3. Electron Microscopic Evidence of Mitochondrial Swelling and Physical Disruption of the Outer Mitochondrial Membrane in Cells Stimulated to Undergo Apoptosis

(A) A transmission electron micrograph of a representative untreated Jurkat cell. The scale bar represents 2 μm.

(B) Representative photomicrograph of a Jurkat cell treated for 6 hr with an anti-Fas antibody. The cell does not yet show any classic morphological features of apoptosis. Nevertheless, the mitochondria are swollen and contain fewer cristae than those in control cells. Several mitochondria display outer membrane discontinuities. The scale bar represents 2 μm.

(C) High-power electron micrograph of the membranes of a mitochondria from a cell treated for 6 hr with an anti-Fas antibody, which, like the cell depicted in (B), demonstrated no chromatin condensation. The matrix side of the membranes is indicated by an m. The arrowhead points to an example of discontinuity in the outer mitochondrial membrane. This particular disruption was observed in three 50 nm thick serial sections. The scale bar represents 0.1 μm.

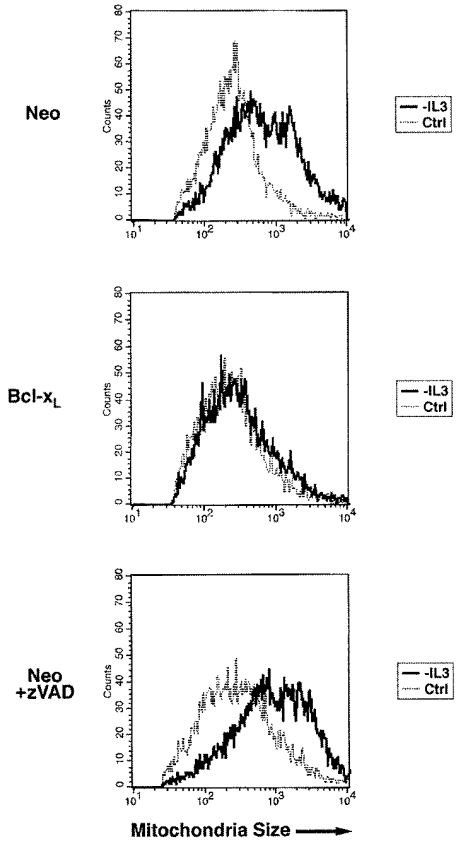

Figure 4. Assessment of Mitochondria Size by Measuring the Forward Scatter of Isolated Mitochondria

Mitochondria were isolated from control- and Bcl-x_L-transfected FL5.12 cells cultured for 18 hr in the presence (Ctrl) or absence (−IL3) of IL-3, with or without zVAD as indicated. The isolated mitochondria were identified by Rh123 fluorescence and size determined by forward angle light scatter using flow cytometry.

not only dependent on the mitochondrial membrane potential ($\Delta\psi$) but also on the volume of the mitochondria. If $\Delta\psi$ is held constant while mitochondria volume increases, a greater amount of Rh123 will be taken up to reach the same internal matrix concentration. Consistent with the EM evidence of mitochondrial swelling, we found that both Jurkat and FL5.12 cells demonstrated an increased Rh123 fluorescence early during the apoptotic response in all forms of cell death examined (Figure 5). The increase in Rh123 fluorescence did not result from an increase in the total cell volume or from changes in plasma membrane potential (data not shown).

The Rh123 fluorescence of untreated control- and Bcl-x_L-transfected cells was similar. However, in response to Fas receptor crosslinking, Jurkat control cells displayed an early increase in Rh123 fluorescence followed by a subsequent loss of that fluorescence over the course of several hours (Figure 5A). Some Bcl-x_L-expressing Jurkat cells underwent similar changes over a more protracted period of time, but a significant percentage of cells maintained the same fluorescence as untreated cells even after 24 hr. In FL5.12 cells following IL-3 withdrawal, the control cells slowly accumulated a population of cells with increased Rh123 fluorescence. This was followed by the appearance of cells that took up substantially less dye (Figure 5B). In both FL5.12 and Jurkat cells, the cells that took up minimal Rh123 were dead as determined by simultaneous measurement of PI permeability (data not shown). FL5.12 cells that expressed Bcl-x_L were protected from death and maintained a decreased but stable Rh123 fluorescence in comparison to cells cultured in IL-3 (Figure 5B). By gating only on live cells (as judged by PI exclusion), increases in Rh123 fluorescence were demonstrated in

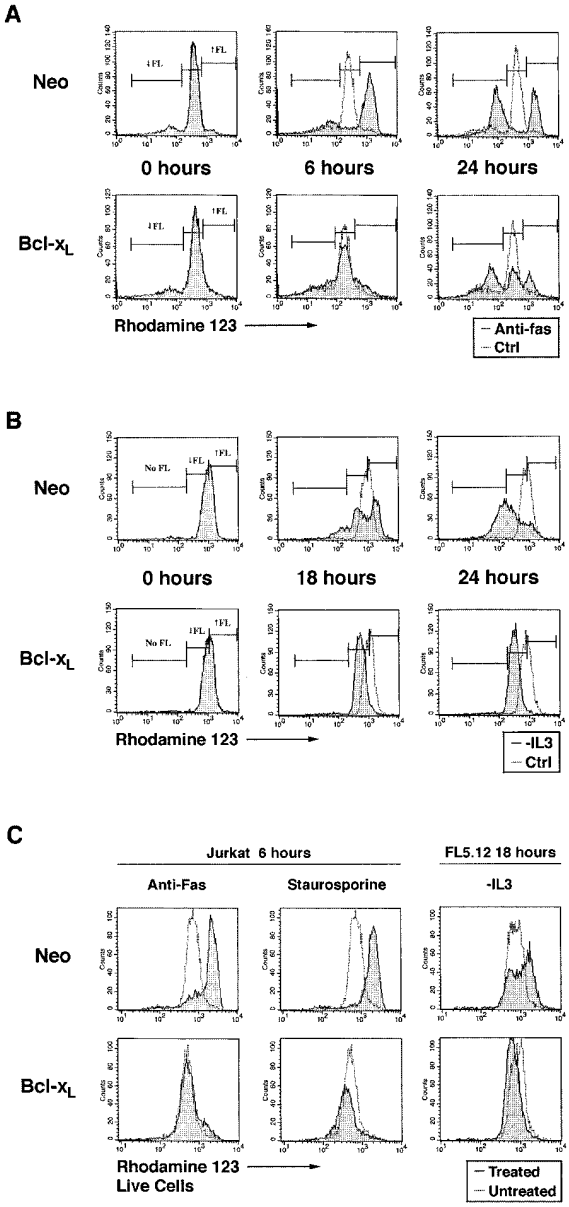

Figure 5. Increased Rh123 Fluorescence Occurs as an Early Cellular Response to Apoptotic Stimuli

(A) Rh123 fluorescence of control-transfected (Neo) and Bcl-x_L-transfected Jurkat cells was analyzed by flow cytometry at the indicated times following anti-Fas treatment.

(B) Bcl-x_L- and control-transfected (Neo) FL5.12 cells were withdrawn from IL-3 and Rh123 fluorescence assayed at the indicated times by flow cytometry.

(C) Jurkat cells were treated for 6 hr with anti-Fas antibody or staurosporine, and FL5.12 cells were cultured for 18 hr following IL-3 withdrawal. Rh123 fluorescence of PI^- (live) cells is shown for both treated and untreated samples.

both Jurkat and FL5.12 cells in response to all of the apoptotic stimuli examined. In contrast, when cells that express Bcl-x_L were exposed to the same stimuli, the live cells maintained a stable and in some conditions decreased level of Rh123 accumulation over time (Figures 5C).

Bcl-x_L Acts to Regulate Mitochondrial Membrane Potential/Volume

Increased Rh123 accumulation could reflect an increase in mitochondrial membrane potential and/or an increase in mitochondrial volume. To examine the relative contributions of mitochondrial membrane potential and volume to the observed changes in Rh123 accumulation, Rh123 fluorescence was measured in the presence or absence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP). CCCP is a protonophore that dissipates the H^+ ion gradient generated by the electron transport chain. This H^+ ion gradient is the major source of mitochondrial $\Delta\psi$ in cells undergoing oxidative phosphorylation.

Treatment of both Bcl-x_L- and control-transfected cells with CCCP demonstrated that Rh123 uptake is dependent on the H^+ ion gradient because CCCP significantly reduces the Rh123 uptake in both cell types (Figure 6). In the presence of CCCP, cells still maintain a basal mitochondrial membrane potential, as judged by dye uptake, that is independent of the proton gradient. Viable control-transfected FL5.12 cells have increased Rh123 uptake following IL-3 withdrawal. Treatment of these cells with CCCP results in a significant decrease in Rh123 fluorescence. However, the residual Rh123 uptake in the presence of CCCP is higher in IL-3-deprived cells than in cells cultured with IL-3. This H^+ ion–independent increase in fluorescence appears to reflect the mitochondrial swelling that occurs in response to IL-3 withdrawal, as the differences in the volume of mitochondria isolated from control- and Bcl-x_L-tranfected cells cultured in the absence of IL-3 (Figure 4) were unaffected by acute addition of CCCP (data not shown). The difference in mean fluorescence intensity with and without CCCP of IL-3-deprived control transfectants was 547 $\pm$ 8 (mean $\pm$ SEM) arbitrary fluorescence units (Figure 6). This difference was slightly larger than the difference of 338 $\pm$ 2 arbitrary fluorescence units observed when the control cells growing in IL-3 were treated with CCCP. Thus, control-transfected cells maintain at least as great a transmembrane potential across their mitochondrial membranes in the absence of IL-3 as they do in the presence of IL-3. In contrast, Bcl-x_L-expressing cells display a lower accumulation of Rh123 in the absence of IL-3, and the Rh123 fluorescence of CCCP-treated, IL-3-deprived Bcl-x_L cells is identical to that of CCCP-treated Bcl-x_L cells growing in IL-3. Therefore, Bcl-x_L cells have a lower H^+ ion–dependent mitochondrial membrane potential in the absence of IL-3 (Figure 6) and do not undergo significant volume changes following growth factor depletion (Figure 4). Treatment of Bcl-x_L-expressing Jurkat cells with staurosporine also resulted in a stable decrease in mitochondrial membrane potential (Figures 1B and 5C). In contrast, viable control transfectants maintain a high membrane potential and undergo progressive mitochondrial swelling in response to staurosporine, even in the presence of zVAD.

The observation of increased fluorescence was not unique to Rh123: similar results were obtained with other cationic dyes that partition to mitochondria, including JC-1 and TMRE. The potential-independent, mitochondrion-selective dye 10N-nonyl acridine orange (NAO) was also examined. NAO accumulates in mitochondria

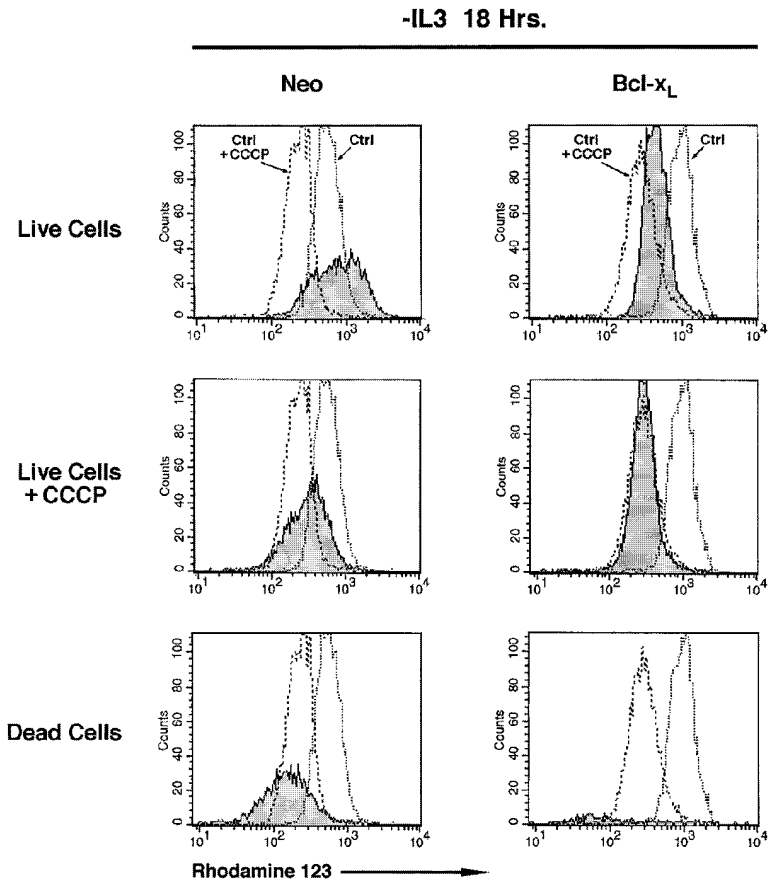

Figure 6. The Increased Rh123 Fluorescence that Precedes Death Is the Result of Both an Increase in Mitochondrial Volume and the H^+ Ion–Dependent Membrane Potential

Control-transfected (Neo) and Bcl-x_L-transfected FL5.12 cells were cultured with or without IL-3 for 18 hr and then incubated with Rh123 in the presence or absence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) prior to being washed and resuspended in PI. PI^- (live) cells cultured in the presence of IL-3 are shown without (Ctrl) and with CCCP (Ctrl + CCCP) for reference purposes in each panel. IL-3-deficient, PI^- (live), or PI^+ (dead) cells, with or without CCCP, are presented as indicated.

by binding to membrane cardiolipin (Petit et al., 1992). At 18 hr following IL-3 withdrawal, control-transfected cells had a slightly decreased NAO fluorescence. In contrast, the NAO fluorescence of Bcl-x_L-transfected cells deprived of IL-3 was unchanged (data not shown). These data suggest that mitochondrial proliferation is not responsible for the increased Rh123 fluorescence observed during apoptosis, nor is a loss of mitochondrial mass responsible for the decreased fluorescence seen in Bcl-x_L-protected cells.

The decreased mitochondrial membrane potential of Bcl-x_L-transfected cells following IL-3 withdrawal could reflect a decreased rate of H^+ ion delivery to the intermembrane space as a result of decreased electron transport, or it could reflect an increased removal of the H^+ ions that are delivered to the intermembrane space. Since the reduction of oxygen by cytochrome oxidase is an essentially irreversible reaction, consumption of oxygen was measured as an index of the rate of electron transport. Both Bcl-x_L and control transfectants exhibited equivalent levels of oxygen consumption in the presence of IL-3 (0.158 ± 0.011 versus 0.168 ± 0.049 μmol O_2/hr $\cdot$ 10^6 cells, respectively). Between 15 and 21 hr following IL-3 withdrawal, both populations decreased their rate of oxygen consumption (38% and 51%, respectively). Analysis using a paired sample t test confirmed that Bcl-x_L-expressing cells maintained a higher rate of oxygen consumption than control cells between 15 and 21 hr following IL-3 withdrawal ($p < 0.05$). Therefore, the lower H^+ ion–dependent membrane potential in Bcl-x_L-transfected cells does not result from a decreased rate of electron transport as compared to control cells. This suggests that Bcl-x_L-expressing cells maintain a lower mitochondrial membrane potential than control cells either by increasing the efficiency of the F_1F_0-ATPase that converts the H^+ ion gradient to ATP or by developing an alternative means to dissipate H^+ ions from the intermembrane space.

Bcl-x_L Prevents Cell Death in Response to Inhibitors of Oxidative Phosphorylation

The previous results suggest that both mitochondrial hyperpolarization and osmotic swelling can be prevented by Bcl-x_L following an apoptotic stimulus. Despite the localization of Bcl-x_L to mitochondria, it is still possible that the protein is functioning to prevent these mitochondrial events by blocking apoptotic signaling pathways before they converge on the organelle. However, a Bcl-x_L construct that lacks the carboxy-terminal membrane anchor, which localizes Bcl-x_L to the outer mitochondrial membrane, displayed a reduced ability to prevent the mitochondrial changes described above (data not shown). To explore this issue further, we tested the ability of Bcl-x_L expression to protect cells following direct perturbations of mitochondrial function. Bcl-x_L- and control-transfected FL5.12 cells were treated with oligomycin or antimycin A. Oligomycin blocks the inner membrane F_1F_0-ATPase, which utilizes the H^+ ion gradient to generate ATP. If H^+ ions are not consumed and electron transport continues, the mitochondria become

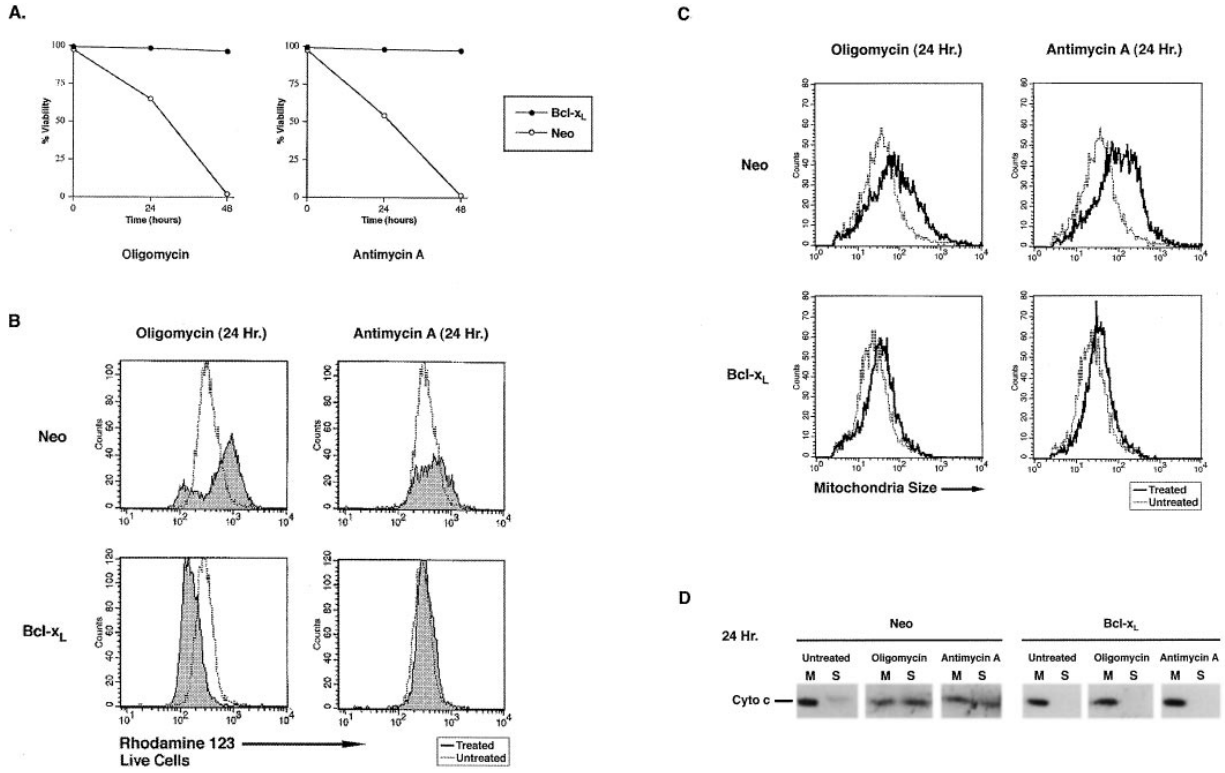

Figure 7. Bcl-x_L Expression Protects Cells Treated with Direct Inhibitors of Oxidative Phosphorylation from Mitochondrial Membrane Hyperpolarization/Swelling and Cytochrome c Release

(A) Control-transfected (Neo) and Bcl-x_L-transfected FL5.12 cells were treated with oligomycin or antimycin A as indicated. At 0, 24, and 48 hr, cells were loaded with Rh123, and nonviable cells were identified by PI uptake.

(B) The Rh123 fluorescence of the live (PI^-) cells at the 24 hr time point of the experiment presented in (A) is shown for each sample as indicated (solid line). The Rh123 fluorescence of live, untreated control-transfected (Neo) and Bcl-x_L-transfected cells are also shown for reference (dashed line).

(C) Assessment of mitochondria size by measuring the forward light scatter of isolated mitochondria. Mitochondria were isolated from cells treated as described in (A) and size determined by flow cytometry as described in Figure 4.

(D) Cells treated as described in (A) were fractionated into mitochondrial (M) and cytosolic (S) fractions as described in Figure 1. The cytochrome c in each fraction was determined by Western blot.

acutely hyperpolarized and subsequently undergo the osmotic swelling associated with necrosis (Figure 7). By 24 hr, when cells were beginning to die, control cells had an increased Rh123 fluorescence, and mitochondria had increased in size in response to oligomycin. Although oligomycin induced a comparable inhibition of oxygen consumption in Bcl-x_L and control transfectants (data not shown), Bcl-x_L expression prevented oligomycin-induced cell death, and the Rh123 fluorescence was decreased. Direct measurement of mitochondria size demonstrated that Bcl-x_L-transfected cells did not undergo significant osmotic swelling. Cytochrome c fractionated with the cytosol in the control transfected cells but not in the Bcl-x_L-protected population.

Antimycin A inhibits complex III of the electron transport chain. Cells treated with antimycin A are unable to maintain mitochondrial osmotic homeostasis following inhibition of electron transport. The mitochondria in antimycin A–treated cells swelled in a manner that is characteristic of cells undergoing necrotic death. Treatment of both Bcl-x_L and control transfectants with antimycin A led to a comparable inhibition of oxidative phosphorylation as measured by oxygen consumption (data not shown). However, as compared to control transfectants, Bcl-x_L expression conferred prolonged protection from death in the presence of antimycin A. Furthermore, Bcl-x_L expression prevented the increase in Rh123 fluorescence, the increase in mitochondria size, and the redistribution of cytochrome c to the cytosol that were observed in control cells following 24 hr of antimycin A treatment.

Discussion

Disruption of mitochondrial function appears to be an early feature of apoptotic cell death. Cytochrome c release and mitochondrial membrane depolarization as a result of the opening of permeability transition pores have both been proposed as early irreversible events during apoptosis. To examine the relationship between these two phenomena and determine their role in the regulation of cell death, we have investigated these mitochondrial events in response to various apoptotic stimuli. Cytochrome c release followed by mitochondrial depolarization occurred early during the apoptotic response to all stimuli tested. However, prior to either of

these disruptions of mitochondrial physiology, an enhanced accumulation of the mitochondrial dye Rh123 was observed. Rh123 accumulation reflected combined changes in the mitochondrial membrane potential and mitochondrial volume. These changes correlated with electron microscopic evidence of mitochondrial swelling and disruption of the outer mitochondrial membrane. Mitochondrial swelling was verified by the direct measurement of the volume of isolated mitochondria. Outer membrane disruption was confirmed by demonstrating that the inner mitochondrial membrane protein, cytochrome oxidase, becomes functionally accessible to cytosolic proteins. Disruption of the outer mitochondrial membrane results in the redistribution of intermembrane space proteins to the cytosol. Ultimately, these events lead to mitochondrial depolarization and cell death.

The above changes appear to define a series of common early events during apoptosis. The results provide a mechanism for the redistribution of cytochrome c and order this event with respect to mitochondrial membrane depolarization. Bcl-x_L expression is able to prevent all of these changes following every apoptotic stimulus tested. Bcl-x_L does not appear to be acting upstream of the mitochondria because it also prevents similar mitochondrial changes following treatment with direct inhibitors of oxidative phosphorylation. In addition, Bcl-x_L-containing mitochondria respond to growth factor withdrawal and metabolic inhibition by the protein kinase inhibitor staurosporine, with a decrease in mitochondrial membrane potential while maintaining oxidative phosphorylation. This response prevents mitochondrial swelling and blocks metabolic depletion of the cell.

Both cytochrome c redistribution and mitochondrial membrane depolarization would be expected to cause cell death. Cytochrome c is a potent activator of caspases in cytosolic extracts (Kluck et al., 1997; Yang et al., 1997). Consistent with this finding, microinjection of cytochrome c results in apoptosis that cannot be inhibited by Bcl-x_L expression (C. S. Duckett et al., unpublished data). In addition, at least one mitochondrial protein released following mitochondrial membrane depolarization has been shown to be an activator of nuclear apoptosis, presumably through caspase activation (Susin et al., 1996). Thus, both the loss of outer mitochondrial membrane integrity leading to cytochrome c release and inner membrane depolarization are caspase-activating events that trigger the apoptotic cascade downstream of Bcl-x_L.

It is likely that loss of outer mitochondrial membrane integrity and the subsequent disruption of mitochondrial function are by themselves events from which the cell cannot recover. Swollen mitochondria are a characteristic feature of necrotic death, and we now demonstrate similar changes in response to apoptotic stimuli. Given these similarities, the question of what distinguishes necrotic from apoptotic cell death is raised. In most necrotic deaths, the loss of oxidative phosphorylation leads to the loss of mitochondrial volume homeostasis (Decker and Wildenthal, 1980). Loss of oxidative phosphorylation also acutely depletes mitochondrial-derived ATP (Lemasters et al., 1987). In contrast, during apoptosis, the inner mitochondrial membrane remains capable of sustaining oxidative phosphorylation despite outer membrane rupture, enabling the dying cell to maintain ATP levels for a prolonged period of time. This may provide energy to fuel the orderly destruction of the cell that characterizes apoptotic cell death. For example, cytosolic ATP and dATP have been reported to be required cofactors of cytochrome c–induced caspase activation (Liu et al., 1996).

Events that destabilize mitochondrial homeostasis during apoptosis may be diverse. For example, the loss of outer mitochondrial membrane integrity may be a direct consequence of matrix swelling. Functioning mitochondria maintain a higher internal osmolarity than the surrounding cytosol. Since the inner mitochondrial membrane has a larger surface area than the outer membrane, upon osmotic swelling of the matrix space, the inner membrane can expand until it physically breaks the outer membrane. IL-3 withdrawal, staurosporine, and inhibitors of oxidative phosphorylation might directly affect metabolic processes that regulate the ability of mitochondria to maintain volume homeostasis. In addition, one can speculate that Fas-induced death may involve a direct proteolytic cleavage of mitochondrial proteins that are directly or indirectly involved in volume homeostasis, while UV irradiation may involve damage to these same protein complexes and/or result in oxidative damage to membrane lipids that increases membrane ion permeability.

Bcl-x_L expression protects mitochondria from the effect of all of these stimuli. In response to several apoptotic stimuli, the effects of Bcl-x_L could not be mimicked by caspase inhibitors, suggesting that Bcl-x_L prevents mitochondrial swelling and outer mitochondrial membrane rupture independent of an ability to inhibit caspase activation. The observation that Bcl-x_L-expressing cells stably maintain a lower mitochondrial membrane potential while sustaining an equivalent rate of oxygen consumption could suggest that Bcl-x_L functions to enhance the efficiency of oxidative phosphorylation. Bcl-2 proteins can also protect cells that lack mitochondrial DNA (ρ^0) and a functional electron transport chain from apoptosis, suggesting that Bcl-2 proteins do not directly exert their effects on the electron transport chain (Jacobson et al., 1993). However, ρ^0 cells still maintain a mitochondrial membrane potential and a hyperosmolar matrix by poorly understood mechanism(s) (Marchetti et al., 1996). Therefore, Bcl-2 proteins may promote the survival of ρ^0 cells by cooperating with the mitochondrial membrane transport systems that are necessary to maintain the high internal osmolarity of mitochondria while preventing osmotic swelling of the matrix.

The ability of Bcl-x_L to modulate the osmotic and electrical homeostasis of mitochondria could be a direct result of its pore-forming properties. For example, Bcl-x_L may form a pore that regulates the accumulation of hydrogen ions in the intermembrane space. Alternatively, Bcl-x_L may function to inhibit other proteins, such as Bax or other Bcl-2 associated proteins, such as a Ced-4 homolog, that can induce mitochondrial damage. Mitochondrial membrane channels and transport proteins are important in the regulation of mitochondria function. For instance, although the molecular composition is unknown, the putative permeability transition pore leads to the osmotic swelling of mitochondria when

it opens (Zoratti and Szabo, 1995). Pharmacological studies have identified the adenine nucleotide translocator (ANT) as a component of this permeability transition pore. Independent investigations have also implicated the ANT in the control of mitochondrial volume (Halestrap, 1989). The ANT cooperates with outer mitochondrial membrane channels such as the voltage-dependent anion channel (VDAC) to regulate the matrix adenine nucleotide content and, consequently, the coupling of oxidative phosphorylation (Adams et al., 1991). It is becoming clear that outer mitochondrial membrane proteins such as VDAC can act cooperatively with inner membrane proteins to regulate the accessibility of both the intermembrane space and the matrix (Adams et al., 1991). Bcl-2 has been reported to localize to such contact sites of the inner and outer mitochondrial membranes (de Jong et al., 1994) and may function to modulate the cooperativity and/or function of proteins that regulate the permeability of mitochondrial membranes.

Experimental Procedures

Cell Lines and Induction of Apoptosis

The Bcl-x_L- and neomycin control vector (Neo)-transfected Jurkat T cells and FL5.12 pro-B cells were cultured as described previously (Boise et al., 1993; Boise and Thompson, 1997). For Fas-induced death, Jurkat cells were cultured in the presence of 100 ng/ml anti-Fas monoclonal antibody (MAb) (Panvera, Madison, WI). Staurosporine (Calbiochem, La Jolla, CA) was used at a concentration of 0.3 μM for Jurkat and FL5.12 cells. FL5.12 cells were deprived of IL-3 by washing the cells three times in media lacking IL-3. Z-Val-Ala-Asp(OMe)-CH_2F (zVAD-fmk) (Enzyme Systems Products, Dublin, CA) was used at a concentration of 50 μM for Jurkat cells and 100 μM for FL5.12 cells where indicated. Both antimycin A and oligomycin (Sigma, St. Louis, MO) were used at 5 μg/ml. Where indicated, cells treated with apoptotic stimuli were fixed and stained for EM as previously described (Robards and Wilson, 1993) and microscopy performed using a JEOL 100CX II electron microscope operating at 60 kV.

Subcellular Fractionation

Mitochondrial and cytosolic (S100) fractions were prepared by resuspending cells in 0.8 ml ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, 2 μg/ml leupeptin [pH 7.4]). Cells were passed through an ice-cold cylinder cell homogenizer (H & Y Enterprise, Redwood City, CA). Unlysed cells and nuclei were pelleted via a 10 min, 750 × g spin. The supernatant was spun at 10,000 × g for 25 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was spun at 100,000 × g for 1 hr. The supernatant from this final centrifugation represents the S100 fraction.

Western Blot Analysis

Equivalent amounts of mitochondrial and S100 fractions were subjected to Western blot analysis performed as previously described (Boise and Thompson, 1997). The primary antibodies were either a 1:1000 dilution of the 7H8.2C12 cytochrome c MAb (kindly provided as a gift by Dr. R. Jemmerson [Minneapolis, MN]) in Tris-buffered saline, 0.2% Tween 20; or 10 ng/ml of 20E8-C12 cytochrome oxidase subunit IV MAb (Molecular Probes, Eugene, OR).

Mitochondrial Membrane Potential and Cell Viability Assays

Cells were incubated in Rh123 (2 μM for FL5.12 and 5 μg/ml for Jurkat cells) (Molecular Probes, Eugene, OR) added to the culture media for 30 min at 37°C. Cells were then washed in PBS before being resuspended in PI solution (2 μg/ml PI in PBS with 1% bovine serum albumin [BSA] and 0.01% azide) to measure cell viability as previously described (Boise et al., 1993). Where indicated, CCCP (Aldrich, Milwaukee, WI) was added at 50 μM with the Rh123. Cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson). To evaluate JC-1 (Molecular Probes) and NAO (Molecular Probes) uptake, FL5.12 cells were incubated in 2 μg/ml JC-1 or 0.1 μM NAO in media for 30 min at 37°C. Cells were washed once, resuspended in PBS, and analyzed by flow cytometry.

Assay for Exogenous Cytochrome c–Dependent Respiration

FL5.12 cells were cultured in the presence or absence of IL-3 for 18 hr as described above then washed in cold PBS. The cells were resuspended in ice-cold MSH buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EGTA, 5 mM succinate, 0.15% BSA, 5 μM rotenone) with 0.01% saponin. The suspension was incubated on ice for 10 min to permeabilize the plasma membrane, then the cells were centrifuged at 450 × g for 5 min at 4°C. The pellet was resuspended in MSH buffer without saponin at a final concentration of 1×10^6 cells/ml. Both cellular oxygen consumption rates and cellular respiration at the cytochrome oxidase site were determined using a previously described procedure (Schumacker et al., 1993; Chandel et al., 1996). The fall in oxygen tension over time (dPO_2/dt) was measured after the sequential addition of 100 μM tetramethyl-p-phenylenediamine (TMPD)/1 mM ascorbate (to provide a nonenzymatic electron donor for cytochrome c) and 10 μM purified bovine cytochrome c (Sigma) to the cell suspension in a respirometer. The exogenous cytochrome c–dependent respiration was determined by subtracting dPO_2/dt measured in the presence of TMPD/ascorbate from that seen following cytochrome c addition. The same procedure was repeated using MSH buffer only to measure the autooxidation of the reagents.

Flow Cytometry of Isolated Mitochondria

A mitochondrial fraction was obtained by mechanical lysis and differential centrifugation and resuspended in ice-cold MSH buffer. Normal goat serum was added to block nonspecific protein interactions. The mitochondria suspension was then incubated with 10 μM Rh123 for 30 min on ice, washed with cold MSH, and resuspended in MSH prior to the determination of forward light scatter by flow cytometry. Alternatively, the mitochondria suspension was incubated with 1 μg of 20E8-C12 anticytochrome oxidase subunit IV MAb (Molecular Probes) or 1 μg of control antibody (R73 antirat TCR MAb, Pharmingen, San Diego, CA) for 15 min on ice. The mitochondria suspensions were washed and resuspended in 100 μl MSH containing 1 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (GIBCO-BRL, Gaithersburg, MD). The mitochondria were stained with secondary antibody for 15 min on ice, washed, and resuspended in MSH for flow cytometric analysis.

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